



Comparative evaluation of immunofluorescent antibody and new immunoblot tests for the specific detection of antibodies against *Besnoitia besnoiti* tachyzoites and bradyzoites in bovine sera

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ABSTRACT

Besnoitia besnoiti, an apicomplexan parasite causes economically important disease in cattle in many countries of Africa and Asia is re-emerging in Europe. Serological identification of infected cattle is important because introduction of these animals into naive herds seems to play a major role in the transmission of the parasite. We report new, simplified immunoblot-based serological tests for the detection of *B. besnoiti*-specific antibodies. Antigens were used under non-reducing conditions in the immunoblots, because reduction of the antigen with β -mercaptoethanol diminished the antigenicity in both, tachyzoites and bradyzoites. Ten *B. besnoiti* tachyzoite and ten bradyzoite antigens of 15–45 kDa molecular weight were recognized by *B. besnoiti* infected cattle, but not or only weakly detected by cattle infected with related protozoan parasites, *Neospora caninum*, *Toxoplasma gondii*, *Sarcocystis cruzi*, *Sarcocystis hominis*, or *Sarcocystis hirsuta*. The sensitivity and specificity of *B. besnoiti* immunoblots were determined with sera from 62 German cattle with clinically confirmed besnoitiosis and 404 sera from unexposed German cattle including 214 sera from animals with a *N. caninum*-specific antibody response. Using a new scoring system, the highest specificity (100%) and sensitivity (90%) of the immunoblots were observed when reactivity to at least four of the ten selected tachyzoite or bradyzoite antigens was considered as positive. When a cut-off based on this scoring system was applied to both the tachyzoite- and the bradyzoite-based immunoblots, there was an almost perfect agreement with the indirect fluorescent antibody test with a titre of 200 as the positive cut-off. We identified and partially characterized 10 tachyzoite and 10 bradyzoite *B. besnoiti* antigens which may help to develop new specific and sensitive serological tests based on individual antigens and in the identification of possible vaccine candidates.

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1. Introduction

Besnoitia besnoiti is a cyst forming apicomplexan parasite closely related to *Toxoplasma gondii* and *Neospora caninum*. It is the cause of bovine besnoitiosis, a severe but usually non-fatal disease with significant economic impact in many countries of Africa and Asia. Bovine besnoitiosis is re-emerging in Europe as recently reported by the European Food Safety Authority (<http://www.efsa.europa.eu/en/scdocs/scdoc/1499.htm>). In acutely infected cattle, bovine besnoitiosis is characterized by pyrexia, nasal and ocular discharge, salivation, stiff gait, and – in severe cases – clinically apparent subcutaneous edema. In chronically infected cattle the skin can become severely lichenified and alopecic. Bulls can develop orchitis which may result in temporary or permanent infertility (Bigalke, 1968). However, usually only few cattle in an infected herd develop typical clinical signs while most animals remain subclinically infected (Bigalke, 1968).

Recently, clinical besnoitiosis was reported in Germany (Mehlhorn et al., 2009; Rostaher et al., in press; Schares et al., 2009; Majzoub et al., 2010). All but one infected animal were born in Germany (Schaes, unpublished observation), indicating that *B. besnoiti* infection can establish in European countries north of the Alps.

B. besnoiti can be transmitted mechanically by tabanids and biting muscids (Bigalke, 1968). Its definitive host is not known (Diesing et al., 1988). Introduction of infected cattle into naive herds seems to play a major role in the transmission of the infection between herds, but also among countries (Bigalke, 1968). Therefore, serological tests are urgently needed to detect infected cattle and to prevent their introduction into non-infected herds.

A number of serological techniques, including immunofluorescent antibody tests (IFAT), enzyme linked immunosorbent assays (ELISA), and immunoblots have been reported for the diagnosis of bovine *B. besnoiti* infection (Neuman, 1972; Shkap et al., 1984; Janitschke et al., 1984; Cortes et al., 2006a; Fernandez-Garcia et al., 2009) but only with limited data on the sensitivity and specificity for some of the tests.

The aims of the present study were to examine the effect of reducing conditions on the reactivity of *B. besnoiti* antigens in immunoblot assays, to develop simple immunoblot-based tests using both tachyzoite and bradyzoite antigens separately and to determine the diagnostic characteristics of these tests. Sera from cattle experimentally infected with other closely related parasites, *N. caninum*, *T. gondii*, *Sarcocystis cruzi*, *Sarcocystis hominis*, or *Sarcocystis hirsuta* were used to detect possible serological cross-reactions. To estimate sensitivity and specificity we used well characterized sera from cattle with clinical besnoitiosis and from uninfected cattle including sera of *N. caninum* seropositive animals. To confirm that the new immunoblot tests are also sensitive in subclinically infected cattle, sera from IFAT positive animals without clinical signs were used, which had been sampled in a herd with confirmed cases of bovine besnoitiosis.

2. Materials and methods

2.1. Bovine sera

2.1.1. Sera from clinically infected cattle

Sera from clinically infected cattle ($n=62$; 8 males, 54 females) were obtained from a herd in Germany where clinical bovine besnoitiosis was first diagnosed in September 2008 (Rostaher et al., in press). All 62 animals had about 0.5 mm large tissue cysts in the scleral conjunctiva of one or both eyes and/or in the mucous membrane of the vestibulum vaginae at clinical examination in November 2008. Blood was taken from a jugular or the tail vein. The sera were used to determine the sensitivity of serological tests for the detection of antibodies against *B. besnoiti*. Fifty-six of these 62 animals were further examined using PCR and histological techniques (Schaes et al., 2009) and besnoitiosis was confirmed by either PCR or histology in 23 animals (Supplementary file Table S1). Eight sera had already been characterized in a previous study on the *in vitro* isolation of *B. besnoiti* (Schaes et al., 2009).

2.1.2. Sera from subclinically infected cattle

Sera from cattle ($n=87$; 7 males, 80 females) with positive *B. besnoiti* IFAT titers of ≥ 100 but no clinically signs from the same herd in Germany with confirmed cases of bovine besnoitiosis were used to confirm the sensitivity of novel immunoblot tests.

2.1.3. Sera from unaffected cattle

Sera of 404 cattle from seven German herds with no history of bovine besnoitiosis were also used. These animals were sampled after *N. caninum*-associated abortions had been diagnosed by histology and PCR in these herds.

2.1.4. Sera from cattle experimentally infected with related protozoans

Sera from cattle experimentally infected with *N. caninum* ($n=2$, 57 days post-inoculation [dpi]), *T. gondii* ($n=2$, 20 or 28 dpi), *S. cruzi* ($n=2$, 41 dpi), *S. hominis* ($n=2$, 43 dpi), and *S. hirsuta* ($n=2$, 34 dpi) (for details, see Schares et al., 1999) were also included.

2.2. Cell culture and purification of tachyzoites

The NC-1 strain of *N. caninum* (Dubey et al., 1988) and the Bb1Evora03 strain of *B. besnoiti* (Cortes et al., 2006b) were maintained in Vero cell cultures and purified as previously described (Schaes et al., 1998, 1999). Purified tachyzoites were used to prepare IFAT slides or pelleted by centrifugation at $1300 \times g$ for 10 min and frozen as a pellet at -80°C until used for immunoblot.

2.3. Isolation and purification of bradyzoites

Lichenified and partially alopecic skin samples collected after slaughtering *B. besnoiti*-infected cattle from the first German bovine besnoitiosis case herd were frozen at -20°C and submitted to the Friedrich-Loeffler-Institut, Wusterhausen, Germany. To remove surface contaminations, the external part of the skin was trimmed away

and the core (1 g) squashed using a mortar and pestle in Dulbecco's Modified Eagle Medium. The parasites were purified with 3 g fibre glass wool (Sigma, Deisenhofen, Germany) filled into a 200 ml borosilicate glass burette. The raw extract (50 ml) was added to the glass wool column and washed with 200 ml phosphate buffered saline (PBS) until 80% of the parasites had passed the column. Eluted parasites were concentrated by centrifugation ($1300 \times g$; 10 min) and frozen at -80°C until used for immunoblotting.

2.4. IFAT

Suspensions of *B. besnoiti* (Bb1Evora03) tachyzoites ($5 \times 10^6 \text{ ml}^{-1}$) in PBS, pH 7.2, were air-dried on glass slides ($10 \mu\text{l}$ /well) and frozen at -20°C until used. The slides were then fixed with ice-cold acetone for 5 min and incubated in PBS for 10 min. Sera diluted in PBS were added for 30 min. The slides were then gently rinsed with fluorescent antibody (FA) buffer (25 mM Na_2CO_3 , 100 mM NaHCO_3 , 35 mM NaCl, pH 9.0) and incubated for 10 min in the same buffer. After a brief incubation in PBS, fluorescein isothiocyanate (FITC) conjugate (anti-bovine IgG [H + L], Jackson ImmunoResearch Laboratories, West Grove, USA), diluted 1:50 in PBS with 0.05% Evans blue, was added. After 30 min incubation, the rinsing and incubation procedure with FA buffer was repeated. After a brief incubation in PBS, anti-fading buffer (3.5 g 1,4 diazobicyclo[2,2,2]-octane [Sigma, Deisenhofen, Germany] in 90 ml glycerol and 10 ml PBS) was added. The slides were examined under an Olympus Vanox AHB3 fluorescence microscope (Olympus, Hamburg, Germany). Only peripheral, but not apical fluorescence was considered specific.

2.5. Preparation of antigen-coated membranes by Western blotting

Samples containing zoites were treated for 10 min at 94°C with non-reducing sample buffer (2% [w/v] sodium dodecyl sulfate (SDS), 10% [v/v] glycerol, 62 mM Tris-HCl, pH 6.8). Aliquots of antigens prepared from 4×10^7 or 4×10^6 zoites were run in a 60 mm or 6 mm wide slot, respectively. In a single experiment 5% [v/v] β -mercaptoethanol was added to this buffer to examine the effect of reduction on antigenicity. The parasite samples were electrophoresed in a SDS-polyacrylamide minigel (12.5%, w/v) along with marker proteins (LMW-SDS Marker Kit, GE Healthcare, Germany) which were run in a separate slot in each gel.

Separated parasite antigens and marker proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Germany) in a semi-dry transfer system (Pharmacia Biotech, Freiburg, Germany), using a current of $1.5 \text{ mA}/\text{cm}^2$ gel for 90 min. The part of the membrane coated with the marker proteins and a 0.5 mm wide strip of the antigen-coated part was cut off and the transferred proteins visualized using an India ink stain (Hancock and Tsang, 1983). The remaining antigen-coated membrane was blocked (30 min, room temperature) with PBS-TG (PBS, 0.05% [v/v] Tween 20, 2% [v/v] fish gelatine liquid [Serva, Heidelberg, Germany]),

air-dried overnight, cut into 60 strips and stored frozen at -20°C until used. Prior to incubation with diluted serum samples, the strips were blocked again with PBS-TG (30 min, room temperature).

2.6. Immunoblot

To detect antibodies against parasite antigens, the incubation of the strips with serum was performed as previously described by Schares et al. (1998) with few modifications. Sera diluted in PBS-TG were incubated with the strips for 1 h at room temperature. To test the effect of antigen reduction, a 1:1600 dilution of the sera was used. To test specificity of the *B. besnoiti* immunoblot with sera from cattle infected with related parasites, a 1:200 serum dilution was applied. The diagnostic immunoblots sera were either diluted 1:100 (in the immunoblot using *N. caninum* antigens) or 1:200 (in the immunoblot using *B. besnoiti* antigens). After washing in PBS-T (PBS, 0.05% [v/v] Tween 20), the strips were incubated with peroxidase conjugate solution (affinity purified goat anti-bovine IgG [H + L], Jackson ImmunoResearch Laboratories, West Grove, USA; diluted 1:500 in PBS-TG) for 1 h at room temperature. After washing in PBS-T and distilled water, antibody reactions were detected by adding substrate solution ($40 \mu\text{l}$ H_2O_2 [30% (v/v)] and 30 mg 4-chloro-1-naphthol [Sigma] in 40 ml PBS, 20% [v/v] methanol). Relative molecular masses were determined by comparison with the LMW-SDS Marker standard. In the *B. besnoiti*-specific tests, pre- and post-infection sera from a heifer experimentally infected with *N. caninum* (Schares et al., 1999) were used as negative controls and sera of three cattle naturally infected with *B. besnoiti* (Cortes et al., 2006a; Schares et al., 2009) served as positive controls. In the *N. caninum* immunoblot, the pre- and post-infection sera from a heifer experimentally infected with *N. caninum* (Schares et al., 1999) were used as negative or positive controls, respectively. In the *N. caninum* immunoblot, reactivity of the sera with non-reduced immunodominant *N. caninum* tachyzoite antigens (NC-IDA) of 17, 29, 30, 33, and 37 kDa relative molecular mass was recorded (Schares et al., 2000). A serum was regarded as *N. caninum*-positive, if at least two NC-IDA were recognized.

2.7. Statistical methods

Test agreement (expressed as Kappa-values), sensitivity and specificity including 95% confidence intervals (95% CI) were calculated using WinEpiscope 2.0 (N. deBlas, C. Ortega, K. Frankena, J. Noordhuizen, M. Thrusfield, <http://www.clive.ed.ac.uk/winepiscope/>).

To compare reaction intensities in immunoblot images, the blots were digitized using a Kodak Image Station 2000MM and analyzed using the ROI tool of the Kodak 1D Image Analysis Software (Eastman Kodak Company, New Haven, CT, USA). ROI lines were placed on each antigen and also between antigen lines to determine the background. The net mean intensity of reaction with each antigen was determined by subtracting the mean background intensity from the intensity measured in each antigen line.

3. Results

3.1. Effect of reduction of antigen by β -mercaptoethanol on the reactivity in immunoblot

Two identical aliquots of tachyzoite or bradyzoite antigens were treated with sample buffer either without or with 5% (v/v) β -mercaptoethanol, separated by SDS PAGE, blotted to PVDF membranes, blocked and probed with the serum of a bull chronically infected with *B. besnoiti* as a positive control. Reactivity was clearly diminished in the antigen preparation treated with β -mercaptoethanol (Fig. 1). To confirm that this is a general effect not only related to a particular control serum, 21 additional sera from chronically infected cattle as confirmed by histology or PCR, were tested in the same way. The plots were digitized and the intensity of reactions analyzed (data in the Supplementary file Table S2). Sera tested with reduced tachyzoite antigens revealed a mean intensity of 52% (SD \pm 28%) of the mean intensity observed in non-reduced antigen. Sera tested with reduced bradyzoite antigens revealed a mean intensity of 47% (SD \pm 27%) of the mean intensity observed in non-reduced antigen. Therefore, it was decided to use non-reduced antigen in all further experiments.

3.2. Identification of specific *B. besnoiti* antigens in the immunoblot

For the identification of putatively relevant antigens visible in immunoblots, sera of eight both clinically and IFAT positive animals (Schares et al., 2009), were examined by immunoblotting with non-reduced tachyzoite or bradyzoite antigens. Antigen bands of potential diagnostic value were pre-selected because these antigens were strongly recognized by at least five of these eight animals. To confirm the specificity of the selected antigens, the tachyzoite and the bradyzoite immunoblots were further tested with sera from animals experimentally infected with *N. caninum*, *T. gondii*, *S. cruzi*, *S. hominis* or *S. hirsuta* (Fig. 2). In both tachyzoite- and bradyzoite-based immunoblots many cross-reacting antigens were observed in bands with relative molecular weights >45 kDa. Therefore, we concentrated on antigens of less than 46 kDa. Ten bands of tachyzoite (45, 40, 37, 34, 30, 27, 22, 17, 16, and 15 kDa) and 10 bands of bradyzoite (41, 36, 33, 28, 26, 24, 23, 22, 20, and 18 kDa) antigens were selected for further validation. These antigen bands yielded no or only very weak cross-reactivity with sera of cattle experimentally infected with *N. caninum*, *T. gondii*, *S. cruzi*, *S. hominis* or *S. hirsuta* (Fig. 2).

3.3. Sensitivity and specificity of an immunoblot to detect antibodies against *B. besnoiti* tachyzoites

Sera from cattle with besnoitiosis, i.e. with demonstrable *B. besnoiti* cysts, recognized up to 10 of the selected tachyzoite antigens. In contrast, sera from herds without besnoitiosis detected only up to three of these antigens (Fig. 3A). The highest specificity was obtained, if reactions with at least four of the tachyzoite specific antigens were regarded as positive. With this cut-off, the tachyzoite-

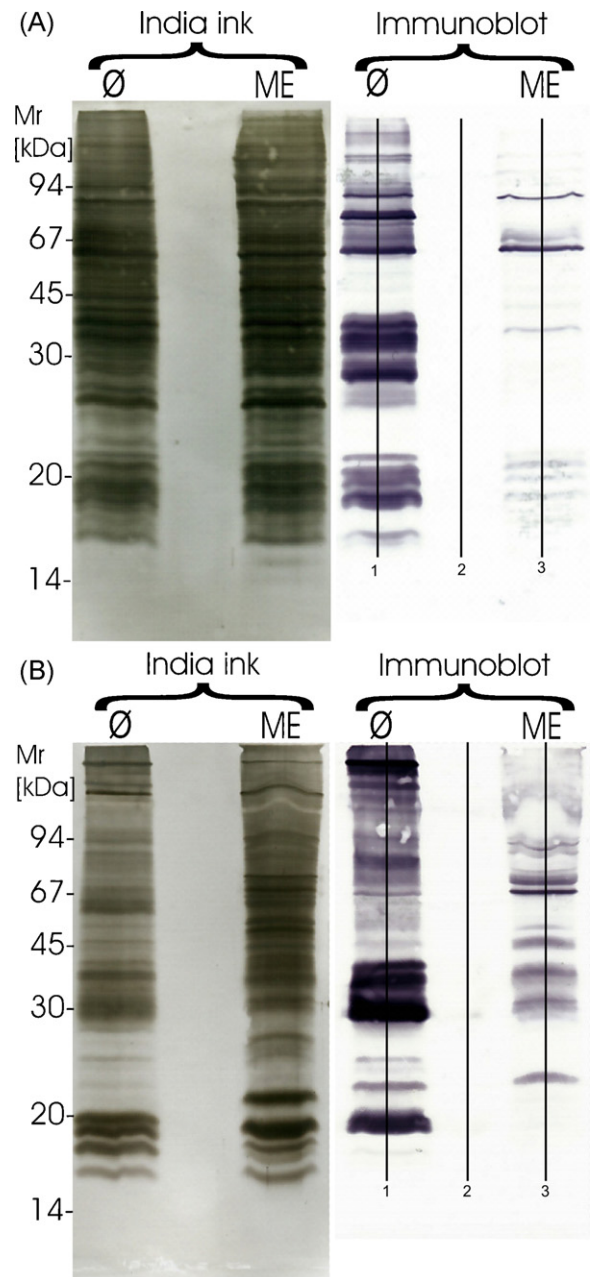


Fig. 1. Two aliquots of 4×10^6 tachyzoites (A) or bradyzoites (B) were treated with sample buffer either without (Ø) or with 5% β -mercaptoethanol (ME), separated by SDS-PAGE, blotted to PVDF membranes, blocked and probed with a positive control serum of a bull chronically infected with *Besnoitia besnoiti*. Fewer bands were detected in the antigen preparation treated with β -mercaptoethanol. Mr = relative molecular weight. Intensity profiles were recorded along the lines for non-reduced and reduced antigens (lines 1 and 3, respectively) and corrected for background (line 2). The mean intensity in the reduced tachyzoite or bradyzoite antigen was 17% or 15% the mean intensity in non-reduced antigen, respectively (for results on additional sera refer to Supplementary data Table S2).

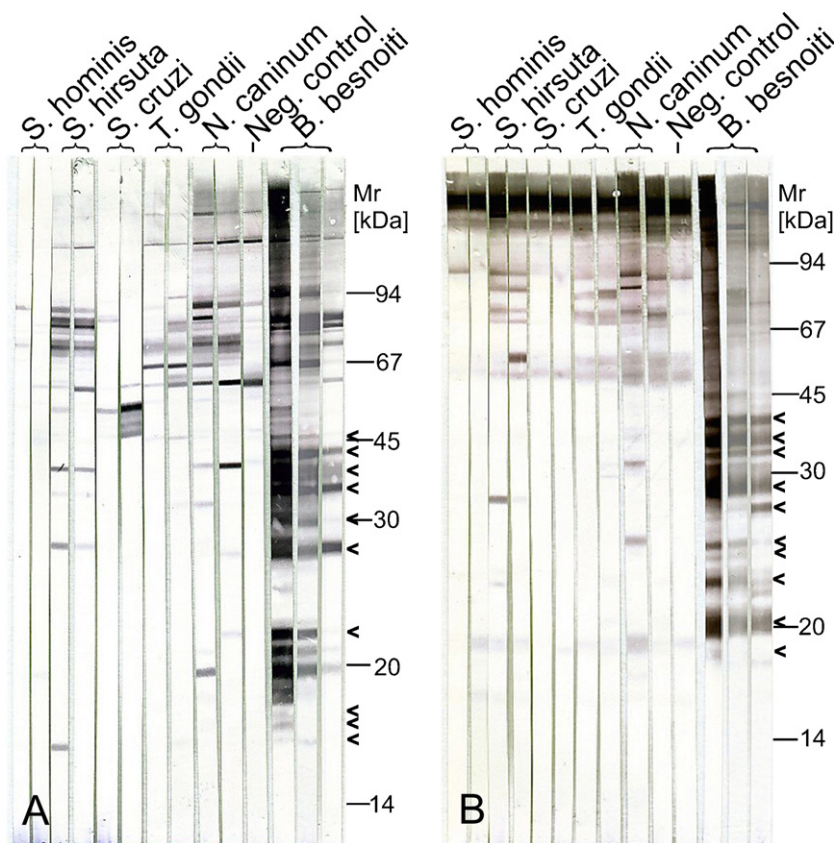


Fig. 2. Immunoblot reactions against non-reduced *Besnoitia besnoiti* tachyzoite (A) or bradyzoite (B) antigens of 10 cattle experimentally infected with *Sarcocystis* (*S.*) *hominis*, *S. hirsuta*, *S. cruzi*, *Toxoplasma gondii* and *Neospora caninum*. The pre-infection serum of one of the *N. caninum*-infected cattle served as a negative control (Neg. control). Sera from cattle with a naturally acquired chronic *B. besnoiti* infection were used as positive controls. Antigen bands selected for scoring the immunoblot reactions are marked (<). Ten tachyzoite antigens (45, 40, 37, 34, 30, 27, 22, 17, 16, and 15 kDa) and 10 bradyzoite antigens (41, 36, 33, 28, 26, 24, 23, 22, 20, and 18 kDa) were selected. Mr = relative molecular weight.

based test had a sensitivity of 90.3% (95% CI: 79.5–96.0%) and a specificity of 100% (95% CI: 98.8–100%). When the cut-off was lowered to the detection of at least three tachyzoite antigen bands, the tachyzoite-based test had the same sensitivity of 90.3% (95% CI: 79.5–96.0%), but a specificity of 98.0% (95% CI: 96.0–99.1%). Three antigen bands of 40,

37 and 27 kDa were recognized by at least 90% of the cattle with besnoitiosis (Table 1). The antigen of 40 kDa was also detected by 14.4% of the cattle from herds without besnoitiosis. The two other antigen bands of 37 and 27 kDa were recognized by 5.0% or 2.2% of the cattle from herds without besnoitiosis.

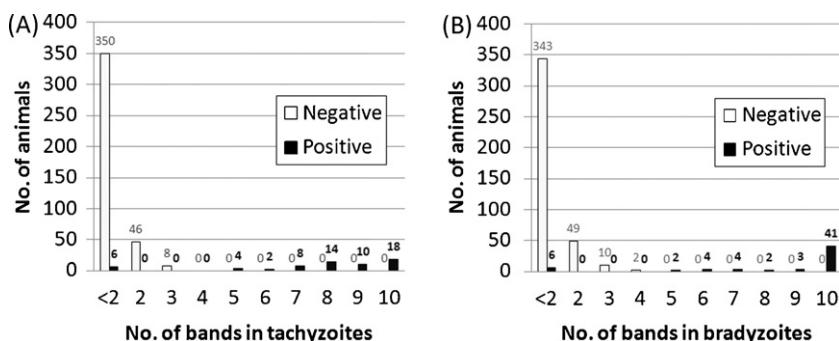


Fig. 3. The numbers of *Besnoitia besnoiti* tachyzoite-(A) and bradyzoite-(B) specific bands observed for 62 German cattle with clinical signs of besnoitiosis (Positive) and 404 cattle from German herds with no signs of bovine besnoitiosis but *Neospora caninum*-associated abortion (Negative). Only positive reactions against 10 selected tachyzoite antigens (45, 40, 37, 34, 30, 27, 22, 17, 16, and 15 kDa) or 10 selected bradyzoite antigens (41, 36, 33, 28, 26, 24, 23, 22, 20, and 18 kDa) were recorded. Figures on top of the bars represent the number of animals that showed reactions with a particular number of bands.

Table 1
Recognition of 10 selected *Besnoitia besnoiti* tachyzoite antigen bands (45, 40, 37, 34, 30, 27, 22, 17, 16, and 15 kDa) by sera from 62 German cattle with clinical signs of besnoitiosis (Positive) and 404 cattle from German herds with no signs of bovine besnoitiosis but *Neospora caninum*-associated abortion (Negative) in the immunoblot.

	Tachyzoite antigen band in kDa relative molecular weight									
	45	40	37	34	30	27	22	17	16	15
Negative (n = 404)	52/404 (12.9%)	58/404 (14.4%)	20/404 (5.0%)	14/404 (3.5%)	11/404 (2.7%)	9/404 (2.2%)	0/404 (0.0%)	4/404 (1.0%)	4/404 (1.0%)	3/404 (0.7%)
Positive (n = 62)	44/62 (71.0%)	56/62 (90.3%)	57/62 (91.9%)	55/62 (88.7%)	51/62 (82.3%)	56/62 (90.3%)	44/62 (71.0%)	43/62 (69.4%)	40/62 (64.5%)	25/62 (40.3%)

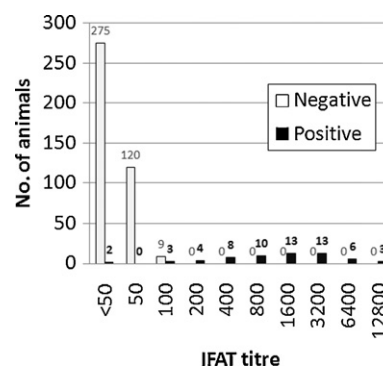


Fig. 4. Anti-*Besnoitia besnoiti* tachyzoite IFAT titres observed in 62 German cattle with clinical signs of besnoitiosis (Positive) and 404 cattle from German herds with no signs of bovine besnoitiosis but *Neospora caninum*-associated abortion (Negative). Figures on top of the bars represent the number of animals that had a particular IFAT titre.

3.4. Sensitivity and specificity of an immunoblot to detect antibodies against *B. besnoiti* bradyzoites

Sera from cattle with besnoitiosis recognized up to 10 selected bradyzoite antigens. In contrast, sera from herds without besnoitiosis detected only up to four of these antigens (Fig. 3B). The highest specificity of the test was obtained, if reactions with at least four bradyzoite-specific antigens were regarded as positive. With this cut-off, the bradyzoite-based test had a sensitivity of 90.3% (95% CI: 79.5–96.0%) and a specificity of 99.5% (95% CI: 98.0–100%). When the cut-off was lowered, i.e. reactions with at least three bradyzoite antigen bands were regarded as positive, the bradyzoite-based test had a sensitivity of 90.3% (95% CI: 79.5–96.0%) and a specificity of 97.0% (95% CI: 94.7–98.4%). Five antigen bands of 41, 36, 33, 28 and 20 kDa were recognized by 90.3% of the cattle with besnoitiosis (Table 2). Two bands of 36 and 33 kDa were also detected by 24.8% and 12.9% of the cattle from herds without besnoitiosis, respectively. The remaining three antigen bands of 41, 28, and 20 kDa, were recognized by 2.0%, 1.7%, or 4.2% of the cattle from herds without besnoitiosis, respectively.

3.5. Sensitivity and specificity of a tachyzoite-based *B. besnoiti* IFAT

The IFAT titres of 62 cattle with demonstrable *B. besnoiti* cysts were <50 in 2, 100 in 3, 200 in 4, 400 in 8, 800 in 10, 1600 in 13, 3200 in 13, 6400 in 6, and 12,800 in 3 animals (Fig. 4). Two animals with *B. besnoiti* IFAT titres of 3200 were also positive in the *N. caninum* immunoblot.

Cattle (n = 404) from herds with *N. caninum*-associated abortion had *B. besnoiti* IFAT titres of <50 in 275, 50 in 120 and 100 in 9 cases; all were negative at titres of 200 or higher. Among these sera, 53% (214/404) yielded a positive *N. caninum* immunoblot result. A statistically significant majority of sera which had *B. besnoiti* IFAT titres of 50 or 100 revealed a *N. caninum*-positive antibody response ($P = 0.025$; Yates corrected Chi-square).

When a cut-off titre of 200 was applied, the IFAT revealed a sensitivity of 91.9% (95% CI: 81.5–97.0%) and a specificity of 100% (95% CI: 98.8–100%). When an IFAT titre

Table 2
Recognition of 10 selected *Besnoitia besnoiti* bradyzoite antigen bands (41, 36, 33, 28, 26, 24, 23, 22, 20, and 18 kDa) by sera from 62 German cattle with clinical signs of besnoitiosis (Positive) and 404 cattle from German herds with no signs of bovine besnoitiosis but *Neospora caninum*-associated abortion (Negative) in the immunoblot.

	Bradyzoite antigen band in kDa relative molecular weight									
	41	36	33	28	26	24	23	22	20	18
Negative (n = 404)	8/404 (2.0%)	100/404 (24.8%)	52/404 (12.9%)	7/404 (1.7%)	1/404 (0.2%)	3/404 (0.7%)	7/404 (1.7%)	12/404 (3.0%)	17/404 (4.2%)	33/404 (8.2%)
Positive (n = 62)	56/62 (90.3%)	56/62 (90.3%)	56/62 (90.3%)	56/62 (90.3%)	50/62 (80.6%)	44/62 (71.0%)	44/62 (71.0%)	51/62 (82.3%)	56/62 (90.3%)	46/62 (74.2%)

of 100 was used as the cut-off, a sensitivity of 96.8% (95% CI: 87.8–99.4%) and a specificity of 97.8% (95% CI: 95.7–98.9%) resulted.

3.6. Comparison of the IFAT and the immunoblot tests

The agreement among all three tests was optimal with Kappa values ≥ 0.95 if all tests were used at a level of 100% specificity (Supplementary file Table S3). Increasing the sensitivity reduced the test specificity to 97.0% or 97.8% and resulted in a decrease in agreement characterized by Kappa values ≤ 0.923 (Supplementary file Table S3). Even when the sensitivity of the tests was increased, six sera from animals with clinical besnoitiosis remained negative in both immunoblots (Supplementary file Fig. S1 and Table 3). Two of these six sera had IFAT titres of 200. When the IFAT cut-off titre was lowered to 100 instead of 200, four of these six immunoblot-negative sera tested positive (Table 3). All sera negative in the immunoblot were from a group of animals with macroscopically demonstrable tissue cysts. However, in these animals the examination of a skin sample revealed a negative PCR or histological result. The six sera which were negative for *B. besnoiti*-specific antibodies by immunoblotting tested also negative for *N. caninum* antibodies.

With a cut-off titre of 200 in the IFAT, five sera of clinically infected cattle tested negative. One of them had an IFAT titre of 100 and was positive in all immunoblot tests. With a cut-off titre 100 in IFAT, only two sera remained negative; application of this cut-off revealed positive reactions also in nine sera from herds with no history of bovine besnoitiosis. Six of these nine false-positive sera tested positive for antibodies against *N. caninum*.

3.7. Sensitivity in subclinically infected cattle

To estimate the diagnostic sensitivity of the immunoblot tests in adult cattle subclinically infected with *B. besnoiti*, cattle without clinical signs but with IFAT titres ≥ 200 or ≥ 100 of the herd affected with bovine besnoitiosis were further examined.

70 of 70 (100%) of the animals with IFAT titres of ≥ 200 recognized at least 3 bands and 69 of 70 (98.6%) at least 4 bands in the tachyzoite immunoblot. 79 of 87 (90.8%) animals with IFAT titres ≥ 100 recognized at least 3 bands and 78 of 87 (89.7%) at least 4 bands in the tachyzoite immunoblot.

All animals (70 of 70, 100%) with IFAT titres of ≥ 200 recognized at least 3 or 4 bands in the bradyzoite immunoblot. 79 of 87 (90.8%) animals with IFAT titres ≥ 100 recognized at least 3 bands and 78 of 87 (89.7%) at least 4 bands in the bradyzoite immunoblot.

4. Discussion

In the present study, the sensitivity of new immunoblots based on non-reduced *B. besnoiti* tachyzoite and bradyzoite antigens was characterized. It was decided to use non-reduced *B. besnoiti* antigens because a reducing buffer in SDS-PAGE destroyed potentially relevant epitopes in both *B. besnoiti* tachyzoites and bradyzoites as already shown for other, closely related parasites (Dubey and Schares,

Table 3

Serological results of 62 German cattle with clinical signs of besnoitiosis in relation to results obtained using a *Besnoitia besnoiti*-specific PCR and/or histological examination.

Polymerase chain reaction/histological result (details in the Supplementary file Table S1)	Number of sera	IFAT positive		Bb-IB-Tachy positive		Bb-IB-Brady positive	
		≥100 ^a	≥200 ^a	≥3 ^a	≥4 ^a	≥3 ^a	≥4 ^a
Positive	23	100%	100%	100%	100%	100%	100%
Negative	33	94%	85%	82%	82%	82%	82%
Not examined	6	100%	100%	100%	100%	100%	100%

PCR = polymerase chain reaction; IFAT = immunofluorescent antibody test; Bb-IB-Tachy = immunoblot with non-reduced tachyzoite antigens; Bb-IB-Brady = immunoblot with non-reduced bradyzoite antigens.

^a Positive cut-offs in the IFAT (titre) or in the immunoblots (minimum number of positive reactions against a set of 10 tachyzoite or 10 bradyzoite bands).

2006). However, this does not imply that immunoblots using reduced antigens have a lower diagnostic sensitivity. Indeed, such immunoblots have recently been described and a sensitive detection of a number of *B. besnoiti* tachyzoite and bradyzoite antigens by clinically and subclinically infected cattle was demonstrated (Fernandez-Garcia et al., 2009).

In the tachyzoite-based *B. besnoiti* immunoblot reported here, we used another scoring system than Cortes et al. (2006a) to examine the results. Cortes et al. (2006a) considered three blocks of antigens for diagnosis, but did not provide data on the size of antigens within these blocks. When sera from cattle infected with *B. besnoiti*-related parasites were tested, reactions observed in all three blocks made a diagnostic decision difficult. To simplify and improve the evaluation process, ten putatively specific bands based on results with sera from cattle infected with *B. besnoiti*-related parasites were identified and the number of bands which had to be recognized to classify an animal correctly as seropositive was then defined.

It was necessary to specify suitable reference populations to determine the diagnostic characteristics of the new tests. To define the positive reference population as precise as possible, we used 62 cattle with clinical signs of besnoitiosis from a herd with a high number of confirmed cases of bovine besnoitiosis. To confirm that the sensitivity of detecting cattle with clinical besnoitiosis was similar to the sensitivity in subclinically infected cattle, 87 sera from the same herd with an IFAT titre of at least 100, but from cattle without clinical signs of bovine besnoitiosis were used, similar to the approach of Fernandez-Garcia et al. (2009).

To estimate specificity, sera from 404 unexposed cattle were employed; about half of these 404 sera showed a specific antibody response in the *N. caninum* immunoblot. Herds with animals suffering from neosporosis were selected in the present study because *N. caninum* is closely related to *B. besnoiti* and cross-reactivity between *B. besnoiti* and *N. caninum* has been reported (Shkap et al., 2002).

At the level of 100% specificity (cut-off: at least 4 of 10 selected bands positive) the tachyzoite immunoblot revealed a sensitivity of 90.3% in cattle with clinical signs. At the level of 98% specificity (cut-off: at least 3 of 10 selected bands positive), the sensitivity remained at 90.3% in infected cattle with clinical signs. This is similar to the results of Cortes et al. (2006a).

In the present study also a new immunoblot test based on non-reduced bradyzoite antigen is reported. Again, 10 specific bands were defined and a scoring system similar to that for tachyzoite antigens was used. At the level of 99.5% specificity (cut-off: at least 4 of 10 selected bands positive), the bradyzoite immunoblot revealed a sensitivity of 90.3% in cattle with clinical signs. At the level of 97% specificity (cut-off: at least 3 of 10 selected bands positive), sensitivity did not increase and remained at 90.3% in infected cattle showing clinical symptoms of besnoitiosis.

It was possible to confirm that both, the tachyzoite and the bradyzoite immunoblot have almost the same sensitivity to detect subclinically infected cattle. In cattle from a herd with confirmed cases of bovine besnoitiosis, which showed *B. besnoiti* IFAT titres of ≥100 but no clinical signs, both immunoblot tests had a sensitivity of around 90% regardless of the cut-off which could be set to a minimum of 3 or 4 recognized bands without a measurable impact on sensitivity. In clinically negative cattle with IFAT titres of ≥200 both immunoblots exhibited an almost perfect sensitivity of 98.6–100%.

Using the panel of sera from cattle with clinical besnoitiosis, also the specificity and sensitivity of the IFAT was determined. At a cut-off of 200, the test yielded a specificity of 100% and a sensitivity of 92% which was slightly better than that of the immunoblots at a level of 100% specificity. With a low cut-off titre of 100, a sensitivity of 97%, i.e. the highest sensitivity of all serological tests used in this study was observed. However, at this cut-off the IFAT had also a reduced specificity of 98%. A statistically significant majority of sera which were false-positive in the IFAT, revealed a *N. caninum*-positive antibody response, suggesting that antibodies against *N. caninum* cross-reacted with the *B. besnoiti* tachyzoites used in IFAT. Cross-reactions between *N. caninum* and *B. besnoiti* were also observed by Shkap et al. (2002) when a cut-off below 200 was applied in the IFAT.

The results of the present study and those of others (Janitschke et al., 1984; Cortes et al., 2006a) highlight the difficulty of serological diagnosis of clinical besnoitiosis. In the present study, *B. besnoiti* antibodies were not detected by immunoblots in six cattle with demonstrable tissue cysts. This was not because we selected inappropriate bands for scoring, since no other prominent antigen bands were detected by these sera, neither with tachyzoite nor with bradyzoite antigen (Supplementary file Fig. S1). The immunoblot of Cortes et al. (2006a) also failed to detect some animals with clinical signs of besnoitiosis.

However, when tested by IFAT, two of the six immunoblot-negative animals had titres of 200 and two others a titre of 100, while the remaining two were negative. This may indicate that the IFAT could be more sensitive than the immunoblots. The observation that two clinically positive animals remained seronegative in three tests could suggest that serologically negative but clinically positive animals may exist in bovine besnoitiosis. Similar observations were made in *T. gondii*-infected pigs in which infection was demonstrated by parasite isolation but not by a positive antibody response in more than one test (modified agglutination test, dye test, ELISA; Dubey et al., 2002; Hill et al., 2006). The reasons why a *B. besnoiti*-infected animal might be clinically positive but seronegative are not known and need to be further analyzed. Perhaps these animals were non-responders due to immuno-tolerance which can occur in foetuses prenatally exposed to the parasite. However, vertical transmission of *B. besnoiti* has so far not been described (Shkap et al., 1994).

In conclusion, two new scoring systems to examine *B. besnoiti* immunoblots based on non-reduced tachyzoite or bradyzoite antigens were established and the diagnostic characteristics of these new tests to examine cattle with chronic besnoitiosis were determined. To our knowledge, this is the first time that the sensitivity and specificity for a test based on *B. besnoiti* bradyzoites have been assessed. With the scoring systems provided in this report, a high specificity was reached in the *B. besnoiti* tachyzoite and bradyzoite immunoblot test. Since immunoblots are difficult to perform and to analyze, these tests are not suitable for routine testing. However, due to their high specificity both assays can be used for confirming results obtained by other tests as, e.g. ELISA and to validate newly developed serological tests. In addition, the immunoblot results in this report may provide important information necessary to develop single-antigen-based serological tests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2010.03.017.

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